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The Utilization of Tissue Cultures for Production of Vaccines Against Venezuelan and American Western Equine Encephalomyelitis Viruses

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Investigations of recent years have shown that the area of spread of the American equine encephalomyelitides is significantly greater than was earlier supposed.

Of particular note are the communications concerning the isolation of the pathogens of the American equine encephalomyelitides in the eastern hemisphere, particularly in Czechoslovakia (6). These data enable us to raise the question about the development of vaccines against the viruses of the American equine encephalomyelitides. The vaccination of laboratory workers is a requirement for work with such contagious agents as the viruses of these diseases.

There is no necessity to emphasize the undoubted advantages of the modern cultural vaccines over the brain and egg (embryo) types.

However, there has not been a single attempt to utilize tissue cultures for the production of vaccines against these viruses, either in the U.S.S.R. nor in those countries where work with these viruses has been conducted for a long period of time and on a broad scale.

Works along the line of specific prophylaxis have been limited to the use of brain vaccines for the vaccination of horses (9) and refined embryo vaccines for the vaccination of laboratory workers (8).

As has been shown in many investigations (2, 4, 5, 7), the American equine

encephalomyelitis viruses propagate in various tissue cultures and build up to high titers in the cultural fluid. Particularly high titers are produced when the viruses propagate in a culture of chick-embryo fibroblasts. Until now, however, the question about the accumulation of the virus in a cultural fluid without protein has not been investigated, although this is extremely important to know in the preparation of a vaccine. Another uninvestigated question is whether the immunogenic property of the virus is retained to an adequate degree in the cultural fluid after processing with formalin.

We set for ourselves the task to learn the possibility of utilizing the tissue culture for the production of vaccines against two viruses: the American western equine encephalomyelitis virus (WEE) and the Venezuelan equine encephalomyelitis virus (VEE).

Materials and Methods

The strain of the WEE virus that we used was put through 45 passages on a culture of chick embryo fibroblasts; the strain of the VEE virus went through 12 passages on a like culture.

A cultural fluid (medium No. 199), which was taken 24 hours after inoculation of the culture, frozen at -70°C and kept at -20°C, was used as the virus-containing material.

A trypsinized suspension of chick-embryo fibroblasts was used for the preparation of the tissue culture. The cells were introduced into the nutrient medium (1.2 million cells to 1 ml of medium). The resultant suspension was poured into separating flasks (100 ml per flask) or into small bottles (1 ml per bottle). A medium of the following composition was utilized: 45% Hanks solution, 45% cow amniotic fluid, 10% ox serum. After a 24-hour incubation at 37°C the cells formed a monolayer. The nutrient medium was drawn off and the culture was inoculated with the virus; after a one-hour contact the virus was drawn off and the supporting medium No. 199 was added.

A neutralisation test was conducted on both mice and on tissue culture; in the latter instance, 0.1 ml of the virus with the serum was added to a like volume of the cellular suspension; after one-half hour the nutrient medium was added. The method for the neutralization on tissue cultures in small bottles has been described in detail previously (1).

Formalin, in an end dilution of 1:4000, was added to the cultural fluid for the inactivation. The incubation was conducted for a period of 5 days at 37°C. A 2.2% solution of sodium bisulfate was utilized for the deformalization (1 ml per 20 ml of vaccine).

The method for checking the immunogenicity of the vaccine is described below.

Results

In the first series of experiments we set our task to determine how the composition of the medium affects the accumulation of the virus in the cultural fluid and whether it is possible to receive high viral titers with the use of a protein-free medium. For this purpose three groups of vials (10 vials per group), containing a monolayer culture of chick-embryo fibroblasts, were inoculated with the WEE virus at 1000 LD_{50} per vial. After a one-hour contact the viral suspension was removed and a supporting medium was added to the vials: in the first group - medium No. 199 without serum; in the second - a medium with a hydrolysate of milk albumin, containing 2% ox serum; and in the third - a medium containing 70% Hanks solution, 20% amniotic fluid and 10% ox serum. This medium, which was borrowed from Brown's work (3), we shall call Brown's medium. Samples of the cultural fluid were taken directly after the addition of the medium and after each 24 hours. The samples were titrated on mice via intracerebral inoculation.

Within 24 hours after inoculation the concentration of the virus in the

cultural fluid had already reached a very high level - near 10^6 LD_{50} in 0.03 ml. This concentration was approximately the same for the vials of all three groups, regardless of the composition of the medium. Later, in the vials where medium No. 199 had been used as the supporting medium the concentration of the virus fell rapidly. Within 48 hours after inoculation it did not exceed $10^{4.5}$ LD_{50} in 0.03 ml. On the other hand, with the use of Brown's medium and especially the medium with the hydrolysate of milk albumin, high viral titers were being detected in the cultural fluid even later than 48 hours after the inoculation (Table 1).

However, not in a single instance was there a higher viral concentration noted than that which is observed within 24 hours after inoculation, while utilizing medium No. 199. Thus, it was proved possible to use a protein-free cultivation medium for the production of vaccine, with the stipulation that the cultural fluid be taken within 24 hours after inoculation.

Before proceeding to the preparation of the vaccine it was necessary to check whether a similar pattern of viral accumulation is observed when working with large volumes of the medium in separating flasks, and whether analogous results are received with the VEE virus. We inoculated two separating flasks (2 for each virus) with the WEE and VEE viruses, with 100,000 LD_{50} per flask. The viral suspension was drawn off after a one-hour contact and 50 ml of medium No. 199 were added to each flask. Titration of samples, taken after 24 hours and 48 hours, confirmed the necessity for using the 24-hour cultural fluid for the production of the vaccine, inasmuch as the titers of the WEE and VEE viruses in it were 10^{-6} and $10^{-6.77}$, respectively; within 48 hours after inoculation the titer of the WEE virus fell to 10^{-5} and the titer of the VEE virus to $10^{-4.5}$.

For the inactivation of the virus, we added formalin, in an end dilution

of 1:4000, to the cultural fluid that had been collected from the flasks within 24 hours after the inoculation. The inactivation was conducted at 37°C. In order to study the pattern of the inactivation, we took samples of the cultural fluid directly after the addition of the formalin, and also after 2 hours, 4 hours and 24 hours. The samples were deformalinized with sodium bisulfate and titrated on mice. The results of these experiments are presented in the diagram. It proved that the WEE and VEE viruses are extremely sensitive to the action of formalin. Within 24 hours after its addition to the cultural fluid the virus could not be detected in the latter. A particularly rapid drop in the titer of the WEE and VEE viruses occurred during the first two hours of inactivation. We conducted the inactivation during 5 days. Then the vaccines were deformalinized. The completeness of the vaccine's inactivation was checked by means of inoculating mice in the brain. Each batch of vaccine (100 ml) was tested on 75 mice (with the exception of the first batch, when only 20 mice were inoculated). After 4 days one-third of the mice were stunned and used for a passage; later, still another passage was conducted. There were 4 batches of the VEE vaccine and 3 batches of the WEE vaccine checked.

In one instance, in the inoculation with the VEE vaccine, one mouse died; inasmuch as we were unsuccessful in isolating the virus from the brain, we evaluate this death as accidental and consider the vaccine as being completely inactivated.

Testing of the immunogenicity of the vaccines was conducted by two methods: by immunization of rabbits and rats with a subsequent determination of the antibody level in the serum, and by means of a direct check of the protective action of the vaccine on mice.

Two rabbits and 5 rats were immunized with each vaccine. The rabbits

were intraperitoneally injected with 5 ml of vaccine three times with an interval of two days. The rats were intraperitoneally injected three times with 2 ml of vaccine with an interval of one day. Within one month after the last immunization the animals were reimmunized a single time, and after two more weeks they were exsanguinated. The antibody content in the sera was determined by means of a neutralization test on mice and on tissue culture. In conducting the test on the mice, tenfold dilutions of the virus were mixed with an equal volume of undiluted serum and, after a one-hour incubation at 37°C, were injected into the brains of the mice in a 0.03-ml volume. The neutralization index was calculated. In the neutralization on tissue culture, four-fold dilutions of the serum were mixed with 100 $TCID_{50}$ of the virus, and after a one-hour incubation, they were introduced into vials containing 0.1 ml of a suspension of chick-embryo fibroblasts. After 30 minutes, Brown's medium was added.

After a 48-hour incubation the results were read according to the cytopathogenic effect. The titer of the serum was determined.

The antibody level in the sera of the immunized animals, particularly in the rabbit sera, proved high. The neutralization indices reached 1000 (table 2).

The titer of the serum from the rabbit immunized with the VEE vaccine proved to be an even 512. There was not a single instance where the neutralization index was below 100, nor where the antibody titer in the serum, which was determined on tissue culture, was below 32.

The immunization of the animals and the subsequent determination of the antibody level in the sera give, however, only an indirect representation of the immunogenicity of vaccines. Therefore we decided to conduct a direct determination of the vaccines' protective strength. Mice, weighing 7-8 gr., received 0.25 ml apiece of the VEE vaccine. It was administered three times intraperitoneally with an interval of one day. Within 10 days after the last injection, the mice were inoculated intraperitoneally with varying dilutions of the VEE virus.

At the same time the control mice which had not received the vaccine, were inoculated with the same dilutions of the virus. The experiment was twice repeated. Mice sucklings were used in an analogous experiment with the WEE virus. The plan and the results of these experiments are shown in table 3. All of the vaccinated mice remained healthy. As seen in table 3, the VEE vaccine insures high indices of protection (more than 1 million). Unfortunately the low sensitivity of the mice to the intraperitoneal inoculation with the WEE virus limits the significance of the resistance test in the work with this virus.

The experimental findings that have been presented indicates the possibility of producing an effective cultural vaccine for the prevention of the equine encephalomyelitides. We do not consider the conditions under which we received our preparation as optimal: we made no attempt to select the best temperature regime during the inactivation period; and we used only one concentration of formalin. All this, however, does not depreciate the fact, but rather emphasizes it, that with the common method of producing a vaccine it is possible to produce an extremely immunogenic preparation that insures the protection of mice from 1 million lethal doses in a intraperitoneal inoculation. This type of vaccine can be recommended for study as a prophylactic agent against the equine encephalomyelitides.

Conclusions

1. The American western and Venezuelan equine encephalomyelitis viruses accumulate in cultural fluid in high titers; with the use of a protein-free medium, however, the viral titer soon drops sharply. It follows, therefore, to use a cultural fluid that has been collected within 24 hours after inoculation for the production of vaccine.

2. The cultural formalinized vaccines against the American western and Venezuelan equine encephalomyelitis viruses cause the appearance of virus-

neutralising antibodies in the sera of vaccinated animals and protect mice from the disease when they are given an intraperitoneal injection of up to 1,000,000 LD_{50} of the virus, thus proving themselves to be highly immunogenic preparations.

Literature

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TABLE 1 CONCENTRATION OF THE WEE VIRUS IN THE CULTURAL FLUID WHILE USING DIFFERENT NUTRIENT MEDIA

VIRAL DOSE FOR THE INOCULATION	TIME LAPSE FROM THE TIME OF INOCULATING THE CULTURE UNTIL THE TAKING OF THE SAMPLES (in days)	THE VIRAL CONCENTRATION IN THE CULTURAL FLUID (LD_{50} in 0.03 ml)		
		Medium No. 199	Medium with the hydrolysate of milk albumin	Brown's medium
10^3 LD_{50}	1	$10^{6.29}$	$10^{5.83}$	$10^{5.5}$
	2	$10^{3.33}$	$10^{5.83}$	$10^{4.5}$
	3	$10^{1.67}$	$10^{5.67}$	$10^{3.0}$
	4	$10^{0.83}$	$10^{3.33}$	Not investigated
	5	-	$10^{2.33}$	-
	6		$20^{2.33}$	

Graph (shown on page 15.)

Inactivation of the WEE and VEE viruses with formalin at 37°C. The time, in hours, is plotted along the horizontal and the logarithm of the viral titer, in LD_{50}/ml, is plotted along the vertical.

TABLE 2 THE CONTENT OF VIRUS-NEUTRALIZING ANTIBODY IN THE SERA OF ANIMALS IMMUNIZED WITH CULTURAL VEE AND WEE VACCINES

SERUM	VIRUS	SERUM TITER IN THE NEUTRALIZATION TEST ON TISSUE CULTURE	NEUTRALIZATION INDEX, WITH THE TEST CONDUCTED ON MICE
Rabbit	VEE	512	$10^{2.27}$
Rat	"	32	$10^{2.11}$
Rabbit	WEE	12[illegible]	$10^{3.14}$
Rat	"	128	$10^{2.44}$

TABLE 3 TESTING OF THE PROTECTIVE ACTION OF THE VACCINES AGAINST THE VEE AND WEE VIRUSES

VIRUS	MICE	DOSE (IP Inoculation)	DILUTION OF THE VIRUS (lg)								INDEX OF PROTECTION
			1.0	2.0	3.0	4.0	5.0	6.0	7.0	8.0	
VEE	Vaccinated	0.25 ml		0/10	0/10	0/10	0/10	0/10			>> 23,710
"	Control	" "				0/10	5/10	7/10	0/10	0/10	
VEE	Vaccinated	" "	0/7	0/7	0/7	0/7	0/7	0/7	0/7		>> 1,000,000
"	Control	" "			7/7	7/7	7/7	8/7	6/7	0/7	
WEE	Vaccinated	" "	0/4	0/4	0/4	0/4	0/4	0/4			
"	Control	" "	4/4	4/4	4/4	2/4	0/4	0/4			>> 3,160 (7)

Designation: numerator = the number of deceased mice.

denominator = the number of mice inoculated.